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28JUN03 E818607-1 D00027
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1. Your reference — 3.81539

2. Patent application number *(The Patent Office will fill in this part)* — 0315131.3 — 27 JUN 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Dynal Biotech ASA
Vor Frue Villa
Ullernchauséen 52
Montebello, Oslo
Norway

Patents ADP number *(if you know it)* 8161655003

If the applicant is a corporate body, give country/state of incorporation — Norway

4. Title of the invention — Improvements in Magnetic polymer particles

5. Name of your agent *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
London
EC4V 4EL

Patents ADP number *(if you know it)* — 166001 ✓

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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Request for substantive examination *(Patents Form 10/77)*	–
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11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 27 June 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Neil Campbell
020 7206 0600

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Improvements in Magnetic Polymer Particles

This invention relates to magnetic polymer particles carrying a chelating matrix loaded with a transition metal as well as to a process for the preparation of magnetic polymer particles carrying said chelating matrix. In particular, the invention relates to magnetic polymer particles carrying a carboxymethylated asparate (Cm-Asp) chelating group and to the coupling of the Cm-Asp group with the magnetic polymer particle.

Magnetic polymer particles are of general utility in various medical and biochemical fields, for example as transport vehicles for the delivery of pharmaceutical products, for diagnostic purposes, for separation and for synthetic purposes. Such particles rely upon their magnetic properties in order to perform these functions: in diagnostic assay applications, for example, application of a magnetic field to a sample containing an analyte bound to magnetic polymer particles allows the isolation of the analyte without the use of centrifugation or filtration; and in therapeutic applications, for example, application of a magnetic field to the patient may serve to target drug-carrying magnetic polymer particles to a desired body site.

By magnetic is meant herein that the polymer particles contain superparamagnetic crystals. Thus the magnetic polymer particles are magnetically displaceable but are not permanently magnetizable. Many processes for preparing magnetic polymer particles are known, a large number of which involve preparing maghemite- or magnetite-containing polymer particles from pre-formed magnetic iron oxides, e.g. magnetite. Some of processes involved are described in US-A-4,654,267 (Ugelstad) the contents of which are incorporated herein by reference.

The use of immobilised metal ion affinity chromatography (IMAC) has been known for many years. The

IMAC purification process is based upon the employment of a chelating matrix loaded with transition metal ions such as Cu^{2+} or Ni^{2+} which is capable of binding electron donating groups present on the surface of proteins, in particular the imidazole side chain of histidine. The electron donating group is believed to coordinate to vacant coordination sites around the metal ion. The interaction between the metal ion and the electron donating groups present on the protein surfaces can be altered by, for example, varying pH and hence purification can be achieved via reversible metal complex/protein interaction. Most commonly, if a protein is bound to a solid phase via the interaction between the metal ion and the imidazolyl side chain of histidine, the protein can be removed by addition of imidazole itself, i.e. by competitive elution.

Several different chelating ligands have been employed in IMAC to purify proteins. Nitrilo triacetate (NTA) (a tetradentate ligand) and the pentadentate ligand tris(carboxymethyl)ethylenediamine are examples of such ligands but these suffer from various disadvantages such as unspecific protein interaction, metal leakage etc.

US 6242581 proposes a solution to the meal leakage problem by the use of a carboxymethylated aspartate (Cm-Asp) group in IMAC where the bound transition metal ion has octahedral geometry. The ligand is said to be ideal for isolating histidine tagged recombinant proteins. Other advantages of Cm-Asp are discussed in US 5962641, e.g resistance to reducing agents.

In these Patents the Cm-Asp ligand is bound to an agarose solid phase which is preferably cross-linked although other polymer matrices such as polystyrene, nylon and SEPHAROSE are suggested. Whilst these matrices may be magnetic the magnetic particles do not remain in suspension and the solid phases are therefore of limited use in assays.

It has now been surprisingly found that the Cm-Asp

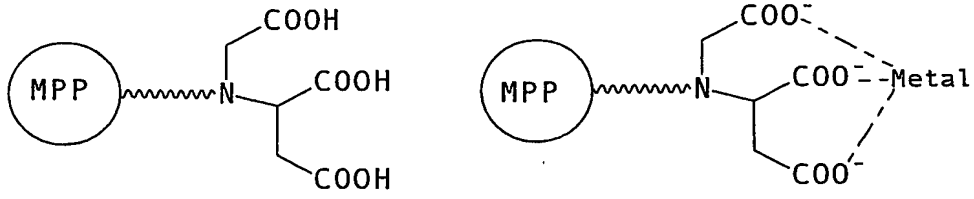
chelating ligand can be coupled to a magnetic polymer particle giving rise to a moiety that possesses not only the ability to bind histidine-tags in recombinant proteins but also magnetism thereby allowing the skilled biochemist more flexibility in his assaying procedures. The inventors have also devised ways to couple the Cm-Asp ligand to the magnetic polymer particles in high yield thereby producing an excellent IMAC agent.

Viewed from a first aspect, therefore, the present invention provides a magnetic polymer particle bound to a carboxymethylated aspartate chelating ligand.

Viewed from another aspect the invention provides a magnetic polymer particle bound to a carboxymethylated aspartate ligand chelating a transition metal ion.

Viewed from another aspect the invention relates to a process for the preparation of a magnetic polymer particle as hereinbefore defined comprising reacting a magnetic polymer particle with a Cm-Asp chelating ligand.

The Cm-Asp ligand bound to the magnetic polymer particle (MPP) is depicted below both in its uncoordinated state and coordinated to a metal ion (the wavy line representing a bond or a linker between the Cm-Asp and particle):



The magnetic polymer particles used in the process of the invention may be any magnetic polymer particle e.g. as described in US-A-4,654,267. The particles are preferably porous to allow the presence of the superparamagnetic crystals in the pores thereof. The surface of the magnetic

particles is normally functionalised to allow coupling of the Cm-Asp ligand to the polymer particle, e.g. it may be functionalised to carry any known surface structure such as carboxyl groups, tosyl groups, amino groups, epoxy groups, maleamido groups, thiol groups etc. Most preferably the surface is amine functionalized before Cm-Asp coupling. Alternatively, a amine functionalised surface can itself be further functionalised to carry other functional groups, e.g. COOH groups.

The polymer particle is preferably made from combinations of vinylic polymers (e.g. styrene), acrylates and/or methacrylates. The polymeric material may optionally be crosslinked, for example by incorporation of cross-linking agents, for example as comonomers, e.g. divinylbenzene (DVB) or ethyleneglycol dimethacrylate. Appropriate quantities of the cross-linking agents (e.g. comonomers) required will be well known to the skilled man. Preferably the polymer is a cross-linked styrenic polymer (e.g. a styrene-divinylbenzene polymer, surface functionalized by the use of a nitro-group containing comonomer, e.g. nitro-styrene, and subsequent reduction) or a cross-linked (meth)acrylic polymer surface functionalized by the use of an epoxy-group containing comonomer (e.g. glycidylmethacrylate) and subsequent amination (e.g. by reaction with ethylene diamine).

The superparamagnetic crystals in the polymer particles used in the process of the invention may be of any material capable of being deposited in superparamagnetic crystalline form in the porous polymer particles. Magnetic iron oxides, e.g. magnetite or maghemite are preferred; however the crystals may be of mixed metal oxides or other magnetic material if desired. The total quantity of crystalline magnetic material present is generally more than 1%, preferably more than 3%, desirably more than or equal to 5% (by weight, e.g. up to 40% wt. The percentage is calculated on a Fe (or

equivalent metal in the case of magnetic materials other than iron oxides) weight basis based upon the overall dry weight of the coated particles.

Polymer particles according to the various aspects of the present invention will generally have sizes (i.e. diameters) that are generally in the micrometer range, e.g. 0.3 to 100 μm, especially 0.5 to 50 μm, more especially 0.8 to 5 μm, e.g. 0.8 to 1.2 μm.

Typically the porous particles used will have a surface area of at least 15 m^2/g (measured by the BET nitrogen absorption method), and more preferably at least 30 m^2/g, e.g. up to 700 m^2/g, when corrected to a mean particle diameter of 2.7 μm (i.e. multiply surface area by 2.7/MD, where MD is the mean diameter in micrometers). Similarly scaled, the particle pore volume is preferably at least 0.1 mL/g.

Typically, the polymer particles are spherical and substantially monodisperse before they are coated and especially preferably remain spherical and substantially monodisperse once they have been coated.

By substantially monodisperse it is meant that for a plurality of particles (e.g. at least 100, more preferably at least 1000) the particles have a coefficient of variation (CV) of less than 20%, for example less than 15%, preferably less than 12%, more preferably less than 11%, still more preferably less than 10% and most preferably no more than about 8%, e.g. 2 to 5%. CV is determined in percentage as

$$CV = \frac{100 \times \text{standard deviation}}{\text{mean}}$$

where mean is the mean particle diameter and standard deviation is the standard deviation in particle size. CV is preferably calculated on the main mode, ie. by fitting a monomodal distribution curve to the detected particle size distribution. Thus some particles below or above mode size

may be discounted in the calculation which may for example be based on about 90% of total particle number (of detectable particles that is). Such a determination of CV is performable on a Coulter LS 130 particle size analyzer.

Functionalisation of the polymeric material may take place after polymerisation by, for example, nitration and subsequent reduction of the thus-formed nitro groups to pendant amine groups; or direct amination, for example by treatment with amino ethanol. As further alternatives, polymeric particles prepared by the well-known Ugelstad two-step swelling process and the improvements thereto disclosed in WO 00/61647 (Dyno) may be used. Porous polymer particles produced according to the processes described in this publication may have magnetic particles deposited in their pores by standard techniques.

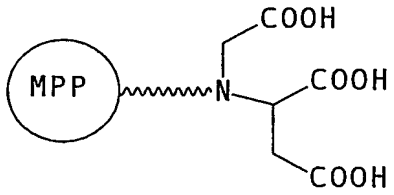
As a further possibility, porous polymer particles may be prepared from nitro styrene and DVB, and magnetic material introduced as taught in US-A-4,654,267.

The superparamagnetic polymer beads sold by Dynal Biotech ASA under the trade names Dynabeads, especially Dynabeads MyOne are especially preferred. Dynabeads are particularly advantageous since they remain in suspension and do not exhibit magnetic particle sedimentation often associated with other magnetic beads. Dynabeads also show excellent magnetic mobility compared to other magnetic particles in which high levels of iron are present. Dynabeads exhibit beneficial kinetics allowing shorter reaction times and higher throughputs. Their unspecified binding is lower than other magnetic beads and their proper use results in a concentration of the desired material taking place resulting in easier and more efficient washing procedures. Finally Dynabeads, e.g MyOne beads are easy to automate and are monodisperse.

The Cm-Asp ligand is bound to the magnetic polymer particle. By bound is meant that the ligand is covalently linked to the polymer particle, optionally using a linking

group as discussed in detail below. The Cm-Asp ligand can be bound to the magnetic polymer particle by various procedures although it is preferred if there are at least three linking atoms between the polymer particle surface and the nitrogen atom of the Cm-Asp, e.g. the styrene surface and the nitrogen atom of the Cm-Asp ligand. Preferably there are at least 6 atoms separating the Cm-Asp ligand from the magnetic polymer particle surface, more preferably there are between 6 and 20 atoms separating the Cm-Asp ligand from the magnetic polymer particle surface.

Hence, in a preferred embodiment the invention provides a composition of formula (I)



(where MPP is a magnetic polymer particle and the wavy line represents a linking group comprising at least three atoms) or an analogue thereof in which a metal ion is chelated.

In US 6242581 aspartic acid is coupled to the solid phase prior to carboxymethylation to form the Cm-Asp ligand however it has not been possible to use this technique to provide a Cm-Asp group on a magnetic polymer particle. Rather, the inventors have devised alternative syntheses in which the Cm-Asp ligand is fully formed prior to coupling to the magnetic polymer particle.

In this regard, it has been found that when there are fewer than 3 atoms between the polymer surface and Cm-Asp ligand then coupling yields are low. In contrast to an agarose support carrying Cm-Asp (as describe in US-A-5962641), it is necessary in the present invention to ensure that coupling yields between the magnetic polymer particle and Cm-Asp are relatively high. The surface area

of an agarose support is considerably greater than that of a polymer particle and hence the binding of Cm-Asp to the support does not need to be achieved in high yield for a useful IMAC chelating agent to result. In the present case, yields need to be much higher to ensure that enough polymer particles carry the Cm-Asp ligand and hence to ensure that IMAC can be successfully carried out.

It is preferred if the at least 3 atom linker comprises an amino group (-NH-). Magnetic polymer beads are often made from styrene polymers which are nitrated to form NO_3 groups on the surface. After reduction of these groups, e.g. using ammonia, amino groups are formed and these form the most common link from the polymer particle surface.

The next portion of the linker preferably represents the residue of an electrophile, i.e. the group which remains after reaction of the electrophile with a nucleophile. Hence, the linker may comprise an oxo group (C=O, the residue of an ester/carboxyl group), a -$CH(OH)CH_2$- group (the residue of an epoxide), -CH_2- (where the electrophile is, for example a CH_2Hal). The linker may also incorporate a number of atoms linking the actual electrophile to the -NH- group, e.g. an alkylene chain or ether chain, e.g. as in -$CH_2CH_2CH_2$-, or -$CH_2CH_2CH_2$-CO-.

A final portion of the linker represents the residue of a nucleophile from the Cm-Asp, i.e. the residue which results after reaction of this nucleophile with the electrophile. As discussed in more detail below this may be a aminoalkylene or aminoether/polyether, thiol or hydroxyl residue.

Hence the wavy line in formula (I) can represent -NH-L_1-Er-Nr-L_2- wherein L_1 represents a 1 to 10 atom linker to the electrophile residue (Er), and L_2 represents a 1 to 10 atom linker to the nucleophile residue (Nr).

It is of course within the scope of the invention for the magnetic polymer particle to carry a nucleophile with

the Cm-Asp being functionalised to carry an electrophilic group.

In a preferred embodiment the polymer particle should be functionalised to carry a coating which can react with the Cm-Asp ligand to couple the magnetic particle to the Cm-Asp.

In an especially preferred embodiment, a particle coating is provided which carries a carbon-carbon double bond. This can be achieved by, for example, reaction of the particle with an allyl or vinyl compound, e.g. butenoic acid. Hydroxy functionalised particle surfaces can be reacted with allyl bromide to form double bonds on the particle surface. Also, carboxy functionalised particle surfaces can be reacted with allyamines to provide double bonds on the particle surface. The Cm-Asp may then be coupled directly to the double bond using appropriate chemistry or more preferably, the double bond may then be reduced e.g. in the presence of aqueous halide to provide a halide electrophile which can be reacted with the Cm-Asp ligand to ensure successful coupling.

Another preferred preparation process involves functionalising the surface of the magnetic polymer particle to carry carboxyl groups. The carboxylic acid groups can be activated by reaction with N-hydroxysuccinimide esters and reacted with a Cm-Asp ligand as discussed above.

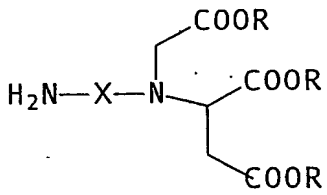
The Cm-Asp ligand can coordinate any transition metal ion although this should preferably be in the 2+ oxidation state. Preferred metals are Ni, Fe, Co, Cu and Zn of which Co, especially Co^{2+} is more preferred. Coordination can be easily effected by exposing the Cm-Asp to, for example, the metal (II) chloride.

The Cm-Asp ligand may too be functionalised prior to coupling with the magnetic polymer particle. For example, it has proved advantageous to provide the Cm-Asp ligand with a linking group carrying a primary nucleophile to aid

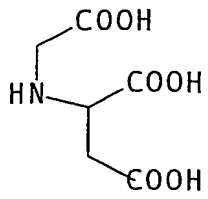
reaction with electrophilic groups on the particle surface. The nitrogen atom of the Cm-Asp ligand is tertiary and it has been found that this atom is too unreactive, perhaps due to steric hindrance, to react in high yield with electrophilic groups, e.g. halides, on the particle surface.

It is preferred therefore to couple the Cm-Asp to a linker group having at least two atoms and comprising a nucleophile such as an amine, hydroxyl or thiol group. Preferably the linker is a alkylamine, e.g. C5/6-alkylamine linker or an ether/polyether linkage e.g. comprising 1 or two oxygen atoms and 3 to 6 carbon atoms. Coupling of the linker to the Cm-Asp (via the nitrogen atom thereof) is achieved using known chemistry as described in the Examples.

Thus viewed from another aspect the invention provides a process for the preparation of a magnetic polymer particle bound to a Cm-Asp ligand comprising reacting a Cm-Asp ligand of formula (II)



(wherein each R independently represents hydrogen or a protecting group and X represents a 2 to 10 atom linker, e.g. an C_{2-10} alkylene linker, especially a $C_{5/6}$-alkylene linker with a magnetic polymer particle functionalise to carry an electrophilic coating, e.g. an ester, epoxide, allyl, alkyl halide etc coating. Compounds of formula (II) are themselves new and form a further aspect of the invention along with the Cm-Asp ligand itself, i.e. a compound of formula (III)



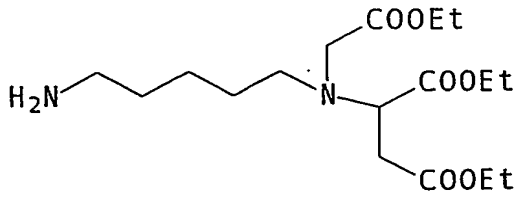
In some embodiments of the invention it may be necessary to protect the carboxyl groups of the Cm-Asp ligand during syntheses. This can be easily effected using known protection strategies, e.g. using an ester protecting group which can be hydrolysed in acid or base as is known in the art.

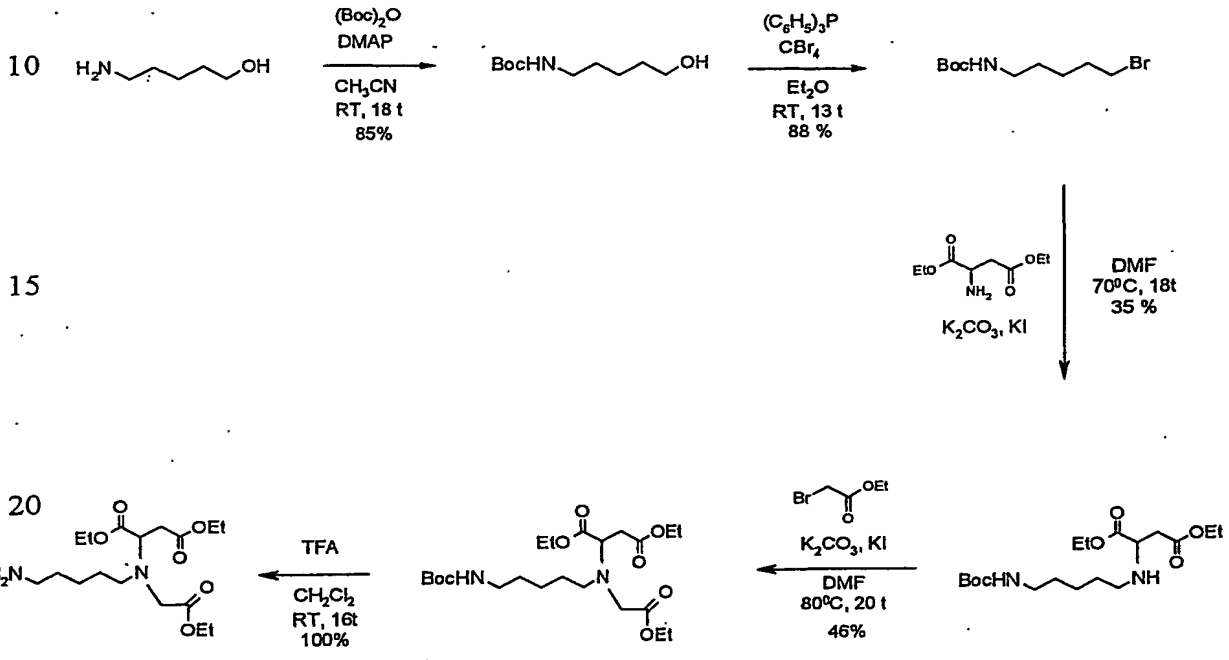
The magnetic polymer particles carrying the Cm-Asp ligand with associated metal ion can be used in a wide variety of assays although they are of particular use in the isolation of His-tags in recombinant proteins. Hence viewed from another aspect the invention provides the use of a magnetic polymer particle bound to a Cm-Asp ligand, said ligand coordinating a transition metal ion, in an assay. Suitable assays and ways to carry these out are known by the skilled biochemist.

The invention will now be described further by reference to the following non-limiting examples.

Reactant Preparation

The Cm-Asp triester below is prepared as follows:





Example 1:

Bromination

17.3 g of a methanol suspension of the magnetic styrene particles having 0.5 mmol/g allyl groups are washed four times with 45 mL sodium acetate buffer (pH = 5.9). After

adjusting the particle content to 9 wt%, 0.96 g of pyridinium tribromide dissolved in 10 mL DMF is added while stirring at 350 rpm. After five minutes at room temperature the particles are washed five times with 45 mL deionised water.

Example 2:

Functionalization with Cm-Asp chelator

18.0 g of a suspension of the particles prepared as in Example 1 are washed three times with 20 mL of 50mM sodium bicarbonate. The particle content is adjusted to 12 wt%. To the suspension 0.17 g of the Cm-Asp triester (prepared as described above) is added. 50mM sodium bicarbonate is added until a particle content of 10 wt% was achieved. The reaction mixture is shaken at 600 rpm at 40°C for 15 hours. The particles are then washed four times with 20 mL deionised water.

Example 3:

Hydrolysis

20.0 g of a suspension of particles prepared as in Example 2 are washed twice with 20 mL of 1M lithium hydroxide. After adjusting the particle content to 10 wt% the mixture is shaken at 250 rpm for four hours at room temperature. The particles were then washed with deionised water until pH 6-7.

Example 4:

Cobalt-loading

250 mg of particles prepared as in Example 3 are washed twice with 5 ml reverse osmosis-water. 5 ml 2,5 mM $CoCl_2$ are added to the particles and incubated for 5 h. The tube

is placed in a magnet, and the supernatant is removed. The particles are washed twice with 5 ml phosphate buffered saline (0,01% Tween 20, pH 7,4). The particles are then washed once in 20% ethanol. The particles are stored in 20% ethanol.

Example 5

Functionalisation of carboxylic acid groups to N-hydroxysuccinimide ester

50 g of a suspension of 5.0 g of the particles of MyOne Carboxylic acid beads are acidified by washing with 0.1 M acetic acid (3 x 50 mL). The acidified particles (which have a carboxylic acid content of 0.5 mmole/g DS) are then washed with acetone (4 x 50 mL) and concentrated on a magnet. Extra acetone is added until a total of 35.6 g suspension is achieved. N-hydroxysuccinimide (2.90 g, 25 mmole) and diisopropylcarbodiimide (3.16 g, 25 mmole) are then added. The reaction mixture is stirred at room temperature for 5 hours. The particles are then washed with acetone (5 x 50 mL).

Example 6:

Functionalization with Cm-Asp chelator

44 g of an acetone suspension of the beads of Example 5, are washed three times with 50 mL isopropanol. After adjusting the particle content to 12 wt%, 5.6 g of triethylamine is added. 0.10 g of the Cm-Asp triester (prepared as described above) dissolved in isopropanol, is then added. This results in a particle content of 10 wt%. The reaction mixture is then shaken at 250 rpm at room temperature for 20 hours. The particles are washed three times with 50 mL of isopropanol.

Example 7:

Functionalization with Cm-Asp chelator and ethanolamine

To 10 g of an isopropanol suspension of the particles prepared as in Example 6, 0.32 g of ethanolamine is added. The reaction mixture is then shaken at 250 rpm at room temperature for 18 hours. The particles are then washed three times with 10 mL of isopropanol.

Example 8

Functionalization with Cm-Asp chelator

1,2 gram of dry Dynabeads 270 Epoxy are mixed with 8,8 gram of 50 mM sodium bicarbonate. To the suspension 0,17 grams of the Cm-ASP triester (prepared as described above) are added, and the reaction mixture is shaken at 600 rpm at 60°C for 16 hours. The particles are worked up by washing four times with 20 ml deionised water.

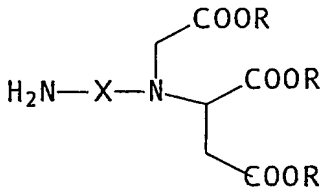
Claims

1. A magnetic polymer particle bound to a carboxymethylated aspartate chelating ligand.

2. A magnetic polymer particle bound to a carboxymethylated aspartate ligand chelating a transition metal ion.

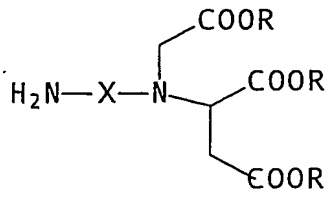
3. A process for the preparation of a magnetic polymer particle as hereinbefore defined comprising reacting a magnetic polymer particle with a Cm-Asp chelating ligand.

4. A process for the preparation of a magnetic polymer particle bound to a Cm-Asp ligand comprising reacting a Cm-Asp ligand of formula (II)



(wherein each R independently represents hydrogen or a protecting group and X represents a 2 to 10 atom linker) with a magnetic polymer particle functionalise to carry an electrophilic coating.

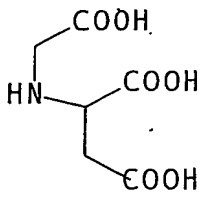
5. A compound of formula (II)



(wherein each R independently represents hydrogen or a

protecting group and X represents a 2 to 10 atom linker).

6. A compound of formula



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